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Short communication

Rapid separation of Sudan dyes by reverse-phase high performance liquid chromatography through statistically designed experiments

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Abstract

Central composite design (CCD) was effectively employed to decide optimum conditions for the rapid separation of Sudan dyes using reverse-phase high performance liquid chromatography (HPLC). Twenty experiments, taking the minimum resolution and retention time of the last eluted peak as the responses with three important factors, mobile phase composition, flow rate and column temperature, were used to design a mathematical model. The experimental responses were fitted into a second order polynomial and used to predict the optimum conditions for the effective separation of the studied compounds. Further, chromatographic separation efficiency was tested through generation of Pareto-optimal points. The validity of using modified central composite design in predicting the optimization conditions was experimentally verified. The optimum conditions were: acetonitrile/0.1% aqueous formic acid (90/10, %v/v) as the mobile phase, at a flow rate of 1.2 mL/min and column temperature of 15 °C, respectively. While using this optimum condition, baseline separation with a minimum resolution more than 1.5 and a separation time of less than 6 min were achieved.

Keywords: Liquid chromatography; Central composite design; Optimization; Sudan dyes; Multi-criteria decision

1. Introduction

People are advised not to eat foods that have been contaminated with illegal Sudan azo-dyes, which are often used for coloring solvents, oils, waxes, petrol, and shoe and floor polishes. The colorant known also as Sudan I–IV (see Fig. 1) have been proved through laboratory experiments [1] to cause cancer to animals and human being. Sudan dyes may cause cancer to people and hence Sudan dyes at any level is not safe for the human. However, Sudan dyes have been found as a contaminant in chilli powder [2]. Soups, sauces and ready meals that use contaminated chilli powder are the main the sources for Sudan dyes. The presence of Sudan dyes has been found in food products and caused panic among customers in China and Europe. Sensitive, selective and accurate analytical methods are to be developed to identify and quantify the Sudan dyes in complex matrices. As reported in the literature, an UV–vis spectrophotometry and a LC–ESI–tandem mass spectrometry (MS/MS) have been employed for the simultaneous identification and determination of Sudan dyes [2,3]. Herein, we employed reverse-phase high performance liquid chromatography (HPLC) for the separation of Sudan dyes and optimized the conditions for rapid separation of Sudan dyes.

Development of effective chromatographic separation method involves judicious selection of experimental conditions that is suitable for the separation of interested components at an adequate resolution with reasonable run time [4]. The analyst must determine the experimental domain, i.e. the variables (composition of the mobile phase, type of column, pH of mobile phase, etc.) that could influence the separation time and efficiency. In addition, an analyst must select the responses (resolution, retention time, etc.) that would give the best resolution

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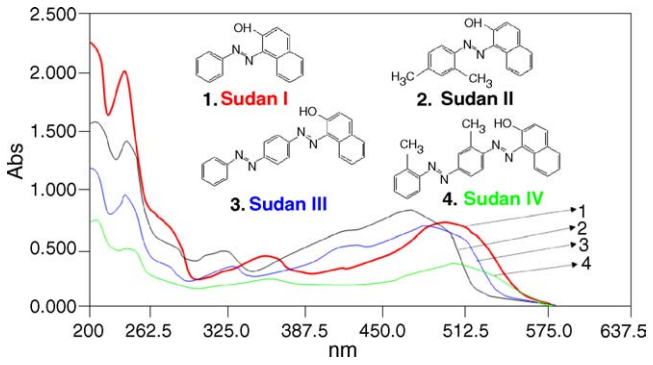

Fig. 1. Scan curves of four Sudan dyes using UV–vis spectrophotometer. Conditions: slit width: 1 nm; scan step: 0.5 nm; scan range: 200–800 nm.

through minimum trail experiments. After taking into account the response assumptions and the variable constraints, the analyst can develop a response model. Consequently, the analyst builds the experimental design that decides the model. Finally, once the experiments are defined and the model is fitted, the analyst tries to achieve the best probable within the experimental domain.

The aim of this study is to develop experimental conditions that would give the best baseline separation for Sudan dyes in HPLC in a relatively short analysis time. Central composite design (CCD) was used to construct mathematical models that predict how changes in input or controlled variables (mobile phase percentage, flow rate, column temperature) affected the two main responses (the last eluted retention time, T_{end}; the minimum resolution, $R_{s,\,min}$) in a defined experimental region. In order to find the best compromise between several responses, Pareto strategies have been employed to simultaneously optimize the selected responses [5]. It is an efficient technique for optimization of multiple variables to predict the best performance conditions with a minimum number of experiments.

2. Experimental

2.1. Materials

Sudan I (1-[(2,4-dimethylphenyl)azo]-2-naphthalenol), Sudan II (1-(phenylazo)-2-naphthol), Sudan III (1-(4-phenylazophenylazo)-2-naphthol), Sudan IV (*o*-tolyazo-*o*-tolylazo-betanaphthol), formic acid and acetonitrile (ACN) were purchased from Beijing Chemical Reagent Company and Tianjing Chemical Reagent Company, China. All solvents used were of HPLC or analytical reagent grade. Distilled water was obtained from a super-purification system (Danyangmen Corporation, Jiangshou, China). All solutions were degassed with ultra-sonication and filtered through a membrane (0.45 μm) before use. In a typical chromatographic experiment, Sudan compound was dissolved in ACN or carbon tetrachloride and injected for peak identification.

2.2. Apparatus

Chromatographic measurements were made on a HP1100 Series HPLC system (AgilentTechnologies, Inc., Walbronn, Germany) equipped with a quaternary pump, a vacuum degasser module, a Rheodyne injector with a 20 μL sample loop, a temperature controlled column compartment and a variable wavelength UV detector set at 276 nm. A HPLC separation was performed on two 15 cm × 4.6 mm I.D., stainless steel columns packed with Zorbax DB-C_8 and Zorbax SB-C_{18} (5 μm particle size, 100 Å (pore size), respectively). Absorbance spectra were recorded using an UV–vis spectrophotometer (Beijing Ruili Analytical Instrument Company).

2.3. Software

Polynomial equations and the statistical analysis of the response variables were performed by Microsoft Excel 2000 software (version 5.0; Microsoft Corp., Redmond, WA). Origin 6.0 (OriginLab Corporation, MA) was used for making the response surface diagrams.

3. Results and discussion

The detection wavelength was chosen by recording the adsorption spectrum of Sudan dye in the range of 200–800 nm using UV–vis spectrophotometry (Fig. 1). The wavelengths in the ranges of 200–260 nm or 450–500 nm were used as the detection wavelengths. In order to decrease the background from mobile phase and the sample's solvent, a wavelength of 476 nm was selected as the most suited wavelength for detection.

Experiments were operated using an isocratic solvent system in ACN/0.1% aqueous formic acid (80/20, %v/v) at a flow-rate of 1 mL/min. Baseline separation of four Sudan samples was obtained using C_{18} column. Fig. 2 informs that a time of 40 min was required for the baseline separation of four components due to the strong retention ability of C_{18} stationary phase towards the hydrophobic compounds. We have developed a statistical strategy to optimize experimental conditions for the rapid separation of the four Sudan dyes.

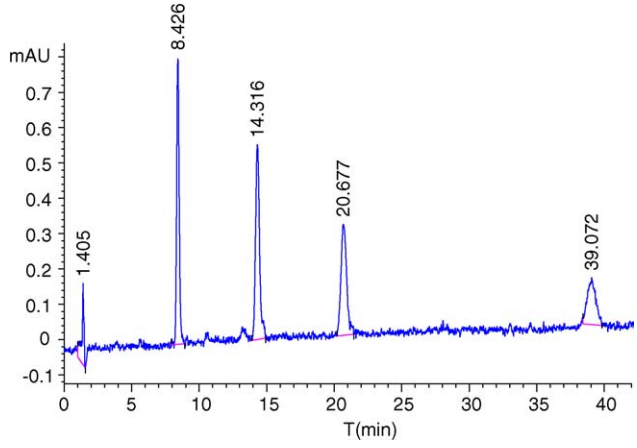

Fig. 2. Chromatogram of four Sudan standards using C18 column. Mobile phase: ACN/0.1% HCOOH = 80/20; stationary phase: ZORBAX SB-C18; injection volume: 20 μL; flow rate: 1 mL/min; temperature: 20 °C; pressure: 31 bar. Peak identification: tR(Sudan) = 8.426 min; tR(Sudan II) = 14.316 min; tR(Sudan III) = 20.677 min; tR(Sudan IV) = 39.072 min.

Table 1
Coded and real values of three variables for CCD model

Level	Percentage ACN (%) x_1	Flow rate (mL/min) x_2	Column temperature (°C) x_3
$-\alpha$	63	0.66	8.2
−1	70	0.8	15
0	80	1.0	25
+1	90	1.2	35
$+\alpha$	97	1.34	41.8

3.1. Experimental design and analysis

CCD is a second-degree design enabling modeling of the nonlinear effect of variables. It was first proposed by Box and Wilson and consists of a full factorial design and a star design [6]. CCD permits to define empirical models (usually quadratic polynomials) that describe accurately how responses behave at all values of the studied variables in the experimental region [7]. In order to calculate the coefficient of quadratic regression model, each design variable has to be at least studied at three distinct levels. Consequently, CCD is often used to provide estimation of a second-order equation, that predicts high efficiency for the analysis with respect to the number of runs required.

Based on preliminary experiments and considering the chemical structure of the studied compounds [3], C_8 column with less retention to the selected analytes was chosen for the optimization. The key variables selected for optimization process were eluent percentage (x_1), flow rate (x_2) and column temperature (x_3). For the selected variables (x_1, x_2, x_3), the center value and the variation step were fixed. The center value and variation step for x_1, x_2, and x_3, respectively, were: 80% and 10% for x_1, 1 and 0.2 mL/min for x_2, 25 and 10 °C for x_3. The experimental matrix at three variables consists of 20 experiments, expressed in coded variables and real variables (x_i) as shown in Table 1. All experiments were performed in randomized order to minimize the effects of uncontrolled variables that may introduce a bias on the measurements. Results of the selected 20 experiments (see Table 2) for the separation of the compounds were effectively used to evaluate the minimum effective resolution (resolution of the least well resolved pair of peaks, $R_{s,\,min}$) and the maximum retention time (for the last eluting peak, T_{end}).

The resolution (R_s) was calculated by using the following formula: $R_s = 2(t_2 - t_1)/(\omega_2 + \omega_1)$, where t_1 and t_2 are the retention times of two adjacent peaks; ω_1 and ω_2, the widths of the two adjacent peaks at the baseline. The experimental responses were then fitted into the following polynomial equation [8–10].

$$Y = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_{12} x_{12} + \alpha_{13} x_{13} + \alpha_{23} x_{23} + \alpha_{11} x_{11}^2 + \alpha_{22} x_{22}^2 + \alpha_{33} x_{33}^2$$

where Y is the response to be modeled ($R_{s,\,min}$; T_{end}); α_i, the coefficients. By using the model, a response surface regression analysis for each response factor was performed by using the SOLVER program in Microsoft Excel. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination (R^2). The coefficients of the response function, their statistical significance, and analysis of variance (ANOVA) for responses were evaluated at the 95% confidence level by the method of least squares (see Table 3). Statistical analysis revealed that the models represented the phenomenon quite well and fitted accurately to the experimental data.

Table 2
Real variables for CCD and experimental responses

S. no.	Percentage ACN (%) x_1	Flow rate (mL/min) x_2	Column temperature (°C) x_3	T_{end} (min)	$R_{s,\,min}$
1	70	0.8	15	39.04	9.95
2	90	0.8	15	8.14	3.02
3	70	1.2	15	26.89	9.80
4	90	1.2	15	5.55	2.79
5	70	0.8	35	26.19	7.72
6	90	0.8	35	5.65	1.45
7	70	1.2	35	17.66	7.56
8	90	1.2	35	3.81	1.34
9	63	1	25	49.25	11.55
10	97	1	25	3.39	0.58
11	80	0.66	25	17.30	5.22
12	80	1.34	25	8.73	5.03
13	80	1	8.2	16.86	7.20
14	80	1	41.8	8.27	3.48
15	80	1	25	11.58	5.29
16	80	1	25	11.58	5.36
17	80	1	25	11.57	5.24
18	80	1	25	11.38	5.08
19	80	1	25	11.38	5.08
20	80	1	25	11.38	5.09

The values of coefficient of determination as 0.994 (in the case of T_{end}) and 0.999 (in the case of $R_{s,\,min}$) showed the reliability of the equation. The ANOVA showed that the percentage of ACN, flow rate and column temperature were significant to retention time of the last eluting peak (p values of <0.0001, 0.0006 and 0.0003, respectively) as expected; for the least resolution, only ACN% and column temperature were significant in the studied domain ($p < 0.0001$). In addition, it was found that the interaction between ACN%–flow rate and ACN

Table 3
Regression coefficients and the associated probability values (p-value) for the retention time of the last eluted peak and the minimum resolution

Term	Retention time		Resolution	
	T_{end} (min)	p-value	$R_{s,\,min}$	p-value
Intercept	588.237	0.000*	55.564	0.000*
x_1	−10.684	0.000*	−0.832	0.000*
x_2	−119.959	0.006*	1.577	0.600
x_3	−2.450	0.003*	−0.270	0.000*
$x_1 \times x_2$	1.016	0.008*	−0.001	0.956
$x_1 \times x_3$	0.022	0.005*	0.002	0.006*
$x_2 \times x_3$	0.274	0.397	0.006	0.820
x_1^2	0.049	0.000*	0.003	0.000*
x_2^2	8.700	0.468	−0.984	0.333
x_3^2	0.002	0.691	0.000	0.352
Multiple R	0.994		0.999	
R^2	0.989		0.999	
Adjusted R^2	0.978		0.998	

Asterisks denote most significant factors and interaction effects (p-value < 0.05).

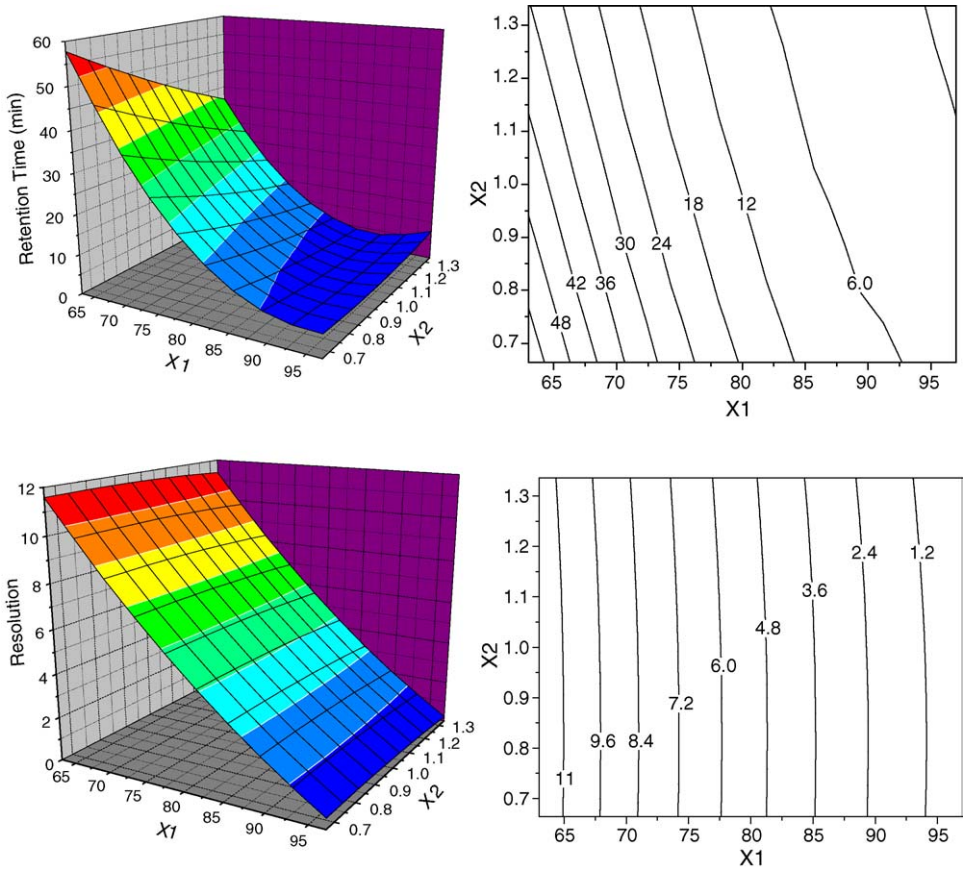

Fig. 3. Response surface of retention time and resolution for mobile phase percentage and flow rate at a constant column temperature of 25 °C; x_1 = %ACN and x_2 = flow rate (mL/min).

percentage–column temperature were also significant with *p*-value of 0.008 and 0.005, respectively, for T_{end}. Whereas, in the case of $R_{s,\,min}$, only ACN%–column temperature was the only significant interaction effect with *p*-value of 0.006. For the quadratic term, only ACN%–flow rate was significant to both responses.

After ascertaining the validity of the model, graphs of surface responses were drawn, by plotting the response variation against two of the variables, while the third variable (center value) was held constant at a specified level. It can be observed that an increased concentration of ACN (x_1) in the mobile phase resulted in decreased T_{end} and $R_{s,\,min}$ apparently, while caused a slight decrease in T_{end} response with the increase of flow rate (x_2). These effects could be seen in Fig. 3. Increasing the column temperature (x_3) resulted T_{end} and $R_{s,\,min}$ to decrease. These effects could be seen from their response surface and contour plots.

3.2. Multi-criteria decision

Generally, responses were usually transformed into an appropriate desirability scale for balance [11]. In that process, different weight variables are to be assigned for each of the response. Larger and smaller weight factors imply more important responses and less important responses, respectively. After obtaining the individual desirabilities for each of the response, they were combined to get a measure of the composite desirability of the multi-response system. This is the measure of the weighted geometric average of the individual desirabilities or the responses [12]. Difficulties may arise in choosing different weights according to the importance of different variables. In such cases, a desirable or compromised condition can be used using Pareto strategies. Interestingly, this strategy does not require any preliminary information. As a result, a compromise between different criteria can be visualized.

The Pareto-optimal plot is depicted in Fig. 4. The three factors were combined to generate 81 combinations with $x_1 \times x_2 \times x_3$.

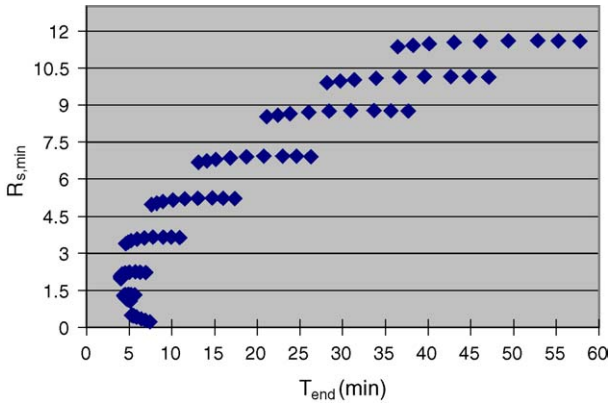

Fig. 4. Plot of the feasible criteria space Pareto-optimality.

Table 4
The comparison of predicted values and experimental values in the optimum range

S. no.	CAN (%)	V (mL/min)	T (°C)	Predicted R_s	Experimental R_s	Predicted T_{end} (min)	Experimental T_{end} (min)
1	80	1.2	25	5.15	4.97	9.45	9.65
2	90	1.2	15	2.84	2.79	5.66	5.56

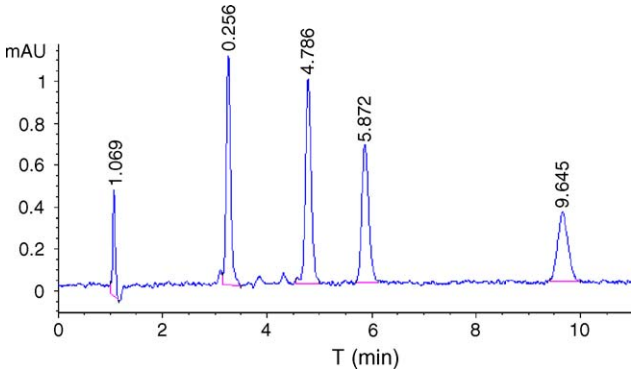

Fig. 5. Chromatogram of four Sudan dyes in the optimal range. Mobile phase: ACN/0.1% HCOOH = 80/20; flow rate: 1.2 mL/min; temperature: 25 °C; stationary phase: ZORBAX XDB-C8; injection volume: 20 μl; wavelength: 476 nm. Peak identification: 0.05 mg/L Sudan I (tR = 3.256 min); 0.05 mg/L Sudan II (tR = 4.786 min); 0.05 mg/L Sudan III (tR = 5.872 min); 0.05 mg/L Sudan IV (tR = 9.645 min).

Among them, x_1 consists of nine levels (63%, 66.5%, 70%, 75%, 80%, 85%, 90%, 93.5%, 97%), x_2 consists of nine levels (0.664, 0.732, 0.8, 0.9, 1.0, 1.1, 1.2, 1.268, 1.336 mL/min), while keeping x_3 at the constant level of 25 °C. It was further decided to optimize the chromatographic separation efficiency based on two objectives: $R_{s,\,min}$ as a measure of the quality of separation and T_{end} as a measure of the cost of analysis. Hence, the minimum effective resolution and the maximum retention time of 81 combinations were visually plotted in the two-dimensional plot (see Fig. 4). The requirements of separations were as follows:

$$T_{end} = 10 \text{ min}; \quad R_{s,min} = 1.5$$

The points (quadrant left-above, see Fig. 4) are called non-inferior combination or Pareto-optimal points. All other points in the feasible criteria space are inferior to these points. The points in the feasible criteria space are Pareto-optimal points (if no other point exists in that space) that can give improvement in any one criterion without affecting the other criterion.

3.3. Method validity

To test the validity of this optimization scheme and to obtain the optimum separation, two optimal points within Pareto-optimal range were selected to perform the experiment. The two points were Pareto-optimal points that fitted the criteria ($T_{end} \leq 10$ min and $R_{s,\,min} \geq 1.5$). This is based on the fact that any point which is in the Pareto-optimal range can be used as the optimal point for verifying the optimization.

The success of the predictions through the modified CCD model in this study can be witnessed in the effective separation of four Sudan dyes using the optimum conditions. A representative chromatograms is presented in Fig. 5. The predicted values agree well with experimental values and support our modified approach to CCD (see Table 4).

4. Conclusion

HPLC offers a quick method for detection and confirmation of the presence of Sudan dyes. Optimization of experimental conditions for the chromatographic system was performed using an experimental design. Further, the experimental data were evaluated with statistical analysis. In summary, few experiments are performed and the models are fitted. It becomes possible to find this best optimum configuration within the experimental domain. The experimental design simplifies the tediousness in the rapid separation of the carcinogenic dye, Sudan.

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